

Neutrophil function and energy status in Holstein cows with uterine health disorders

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Abstract

The objectives of this study were to investigate the associations between peripheral blood neutrophil (PMN) function, energy status, and uterine health in periparturient dairy cows. Data were collected from 83 multiparous Holstein cows. Blood samples for PMN function determination were collected weekly from 1 week prior to calving (week –1) through 4 weeks after calving and again at 8 weeks after calving. Energy metabolites were measured and dry matter intake (DMI) was determined from weeks –2 to 5 to evaluate energy status of cows during the periparturient period. All cows were examined for uterine health disorders. Blood PMN killing ability was evaluated by determining myeloperoxidase activity and cytochrome *c* reduction activity in isolated blood PMN's. For cows that were diagnosed with puerperal metritis and subclinical (SC) endometritis and puerperal metritis, blood PMN functions were significantly ($P < 0.05$) impaired during the periparturient period, compared to cows with normal uterine health. Cows with subclinical endometritis and puerperal metritis or SC endometritis also had significantly ($P < 0.01$) higher NEFA and significantly ($P < 0.001$) lower DMI during the periparturient period, and significantly ($P < 0.05$) higher BHBA during early lactation, compared to cows with normal uterine health. Neutrophil function was also significantly ($P < 0.01$) impaired in cows with peripartum negative energy balance, which was characterized by elevated blood levels of NEFA and decreased DMI. Decreased PMN function and energy balance were associated with uterine health disorders and the decreases in PMN function and energy balance occurred prior to parturition and prior to the detection of these uterine disorders. © 2006 Elsevier B.V. All rights reserved.

Keywords: Periparturient diseases; Neutrophils; Endometritis; Dairy cow

1. Introduction

Maximizing reproductive efficiency is the goal of reproductive management programs in dairy herds. Infection and subsequent inflammation of the bovine uterus compromise uterine health and contribute to decreased reproductive efficiency in dairy cows

Abbreviations: TMR, total mixed ration; NEFA, non-esterified fatty acids; BHBA, β -hydroxybutyric acid; DMI, dry matter intake

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(Coleman et al., 1985; Fourichon et al., 2000). Metritis and endometritis (LeBlanc et al., 2002) are prevalent in dairy herds and contribute to increased days to first breeding, decreased conception rate and pregnancy rate, and increased culling. Subclinical endometritis, based on uterine cytological examination, is also prevalent in dairy cows and has a profound negative impact on reproductive performance. Gilbert et al. (2005) and Hammon et al. (2001) reported that cows with subclinical (SC) endometritis had lower conception rates and higher reproductive failure rates, compared to cows without SC endometritis. More recently, Kasimanickam et al. (2004) showed that cows with SC endometritis, based on endometrial cytology examination at 34–47 DMI, had significantly lower first service and all service conception rates compared to cows without endometritis.

Peripheral blood neutrophil (PMN) function of periparturient dairy cows is impaired relative to non-parturient cattle (Kehrli et al., 1989; Cai et al., 1994). Blood PMN function begins to decline prior to parturition, reaches a nadir shortly after parturition, and slowly returns to prepartum levels by about 4 weeks postpartum (Kehrli et al., 1989). Studies have shown a relationship between periparturient PMN function suppression during the periparturient period and retained placenta (RP) (Gunnink, 1984; Kimura et al., 2002) and metritis (Cai et al., 1994) in dairy cows. Neutrophils from cows with RP have decreased migration ability (Gunnink, 1984) and decreased myeloperoxidase activity (Kimura et al., 2002). Blood neutrophil functions in periparturient dairy cows with SC endometritis have not been reported.

Decreased DMI prior to parturition is well documented and is associated with mobilization of lipids, which are released as NEFA from adipose tissue (Grummer et al., 2004). Decreased DMI and increased NEFA levels are temporally associated with periparturient immune function suppression and may contribute to impairment of the immune system in dairy cows (Rukkwamsuk et al., 1999). Furthermore, elevated levels of β -hydroxybutyric acid (BHBA) and other ketones have been shown to impair important functions of immune cells with possible implications for systemic infections postpartum (Klucinski et al., 1988). Kremer et al. (1993) reported that cows in negative energy balance (with elevated BHBA) prior to experimental infection of the mammary gland

experienced more severe mastitis compared to cows with low blood BHBA concentrations, indicating that negative energy balance may predispose cows to severe infections. Hoeben et al. (1997) reported that exposure of PMN's to elevated levels of BHBA reduced PMN respiratory burst and they concluded that BHBA may, in part, be responsible for the higher susceptibility to local and systemic infections during the postpartum period. There is also evidence of an association between elevated levels of milk ketones (acetone) and endometritis in dairy cows (Reist et al., 2003). However, interrelationships between peripartum energy status, PMN function, and uterine health are not well understood.

We hypothesize that impaired PMN function and negative energy balance are associated with and occur prior to the onset of uterine health disorders in dairy cows. The objectives of this study were to evaluate the interrelationship between uterine health disorders, energy status, and PMN function in periparturient dairy cows.

2. Materials and methods

2.1. Animals

Multiparous Holstein dairy cows ($n = 83$) from the Utah State University, George B. Caine, Dairy Teaching and Research Center were used for this study. Care and use of all cows in this study were in accordance with approved IACUC protocol (USU 1154). Cows were housed in ties stalls and individually fed a total mixed ration (TMR) twice daily and water ad libitum. A close-up diet was fed beginning 3 weeks prior to expected calving date, a fresh cow diet was fed beginning the day of calving through 3 weeks postpartum, and a lactation diet was fed from 3 weeks postparturition to the conclusion of the study (Table 1).

Table 1
Chemical composition (dry matter) of diets for cows in three stages of lactation

Chemical composition	Close-up	Fresh	Lactating
NE _L (Mcal/lb)	1.49	1.54	1.63
CP (%)	13.7	17.7	18.2
NDF (%)	42.1	39.6	38.5
ADF (%)	26.2	28.2	27.8

2.2. Uterine health and treatment

Cows were examined daily and peripartum disorders and diseases were recorded. Rectal temperatures were taken daily in the morning and recorded beginning the day of calving until 10 days postpartum. Cows with fetid red-brown watery uterine discharge between 0 and 14 days after parturition, regardless of presence of fever, were classified as having puerperal metritis. All cows classified as having puerperal metritis were treated with ceftiofur hydrochloride (2.2 mg/kg) for at least 3 days or, if fever was present, until rectal temperature returned to normal. Cows with rectal temperatures greater than or equal to 39.5 °C for two or more consecutive days were classified as having fever. Vaginal speculum and endometrial cytological examinations were performed on day 21 (± 3) and 28 (± 3) days postpartum, respectively. Cows with purulent discharge at the external o.s. of the cervix were classified as having endometritis and cows with >25% neutrophils on cytological examination were classified as having subclinical (SC) endometritis.

2.3. Endometrial cytological examination

Endometrial cytological samples were collected to determine the presence or absence of SC endometritis at 28 (± 3) days postpartum. Endometrial cytological samples were obtained by uterine lavage. Uterine lavage was performed by placing a sterile 22 in. disposable infusion pipette connected to a 20 ml syringe and depositing 15 ml of sterile saline into the uterine body. The lavage fluid was then agitated via rectal manipulation to dispense the solution from the uterine body into the uterine horns. Lavage fluid was then aspirated from the uterine body into the syringe and placed into a 15 ml conical centrifuge tube. Endometrial cytology samples were then centrifuged at $500 \times g$ for 5 min. Centrifugation yielded a cell pellet at the bottom of the tube. A small sample from the pellet, consisting of endometrial cells and/or PMN's, was pipetted onto a clean glass microscopic slide, and a thin layer smear was prepared. Endometrial cytological preparations were stained, evaluated, and classified as described by Kasimanickam et al. (2004).

2.4. PMN function assays

Blood samples for PMN function assays were collected via jugular vein venipuncture at 1 week prepartum (week -1), the week of calving (week 0), and at 1–4 and 8 weeks postpartum for PMN function determination. Fifty ml of blood from each cow were collected and transferred into a sterile centrifuge tube containing 5 ml of anticoagulant (4.4% trisodium citrate, 1.6% citric acid, 5% dextrose, pH 6.1). The samples were shipped overnight to the Metabolic Diseases and Immunology Unit, National Animal Disease Center, Ames, IA, for analysis. Blood collected on the same day from two mature Holstein steers housed at Utah State University served as internal laboratory standards for PMN function assays. Myeloperoxidase activity and cytochrome *c* reduction assays were performed on each blood sample at each time period. Blood PMN's were isolated as described by Roth and Kaeberle (1981). Briefly, each blood sample was centrifuged for 20 min ($1000 \times g$) and the plasma and buffy coat aspirated and discarded. PMN were separated from the remaining packed RBC by using a two volume hypotonic lysis. The PMN cells (primarily neutrophils, but also includes basophils, and eosinophils) were adjusted to a final concentration of 5.0×10^7 PMN's/ml in Hanks Balanced Salt Solution (Grand Island Biological Co., Grand Island, NY) at room temperature until function was assayed. The evaluation of PMN function was accomplished using two indices of the ability of PMN to kill bacteria.

Once immunoglobulins or complement components bind receptors on the surface of PMN to initiate phagocytosis the cell becomes activated, which typically involves a large increase in oxygen consumption and hexose monophosphate activity with subsequent generation and release of large amounts of superoxide anion to the cell surface and to the phagosome which then spontaneously converts to hydrogen peroxide. These compounds alone are toxic to many bacteria but as the phagosome fuses with primary granules of the neutrophil, myeloperoxidase is released. Myeloperoxidase catalyzes the reaction between hydrogen peroxide and chloride (and other halide) anions to form hypochlorite. Hypochlorite reacts with tyrosine and other residues of bacterial proteins to kill bacteria. We utilized a cytochrome *c* reduction assay to measure the amount

of superoxide anion produced by PMN during the oxidative metabolic burst associated with phagocytosis of pre-opsonized zymosan. We then determined myeloperoxidase activity by measuring the ability of PMN to convert inorganic iodide to a trichloroacetic acid precipitable protein bound form following activation of the PMN by pre-opsonized zymosan.

The cytochrome *c* reduction assay was performed as described by Canning et al. (1986). Each cytochrome *c* reduction assay was performed in triplicate and the average value was used in the final analysis. The myeloperoxidase activity (iodination) assay was performed as described by Roth and Kaeberle (1981). Each iodination assay was performed in duplicate and the average of the two values was used in the final analysis. The PMN function data is reported as percentage of response of PMN's obtained from subject animals, compared to the response of PMN's obtained from the internal laboratory standard (steer samples) obtained at the same time.

2.5. Energy metabolites and dry matter intake

Blood samples for NEFA and BHBA were collected via the coccygeal vein twice weekly, 4–5 h after morning feed was offered, from 2 weeks prepartum until 5 weeks postpartum. Weekly mean NEFA and BHBA concentrations were used in the final analysis. Plasma was frozen at -20°C until analysis was performed in bloc.

Plasma NEFA concentrations were determined colorimetrically (Johnson and Peters, 1993) using the Wako NEFA C kit (Wako Chemicals USA, Richmond, VA). Each analysis was performed in triplicate and the average value used. Cows with NEFA ≥ 0.400 mequiv./l prior to parturition were classified as having elevated NEFA.

A colorimetric assay for BHBA concentration in plasma was performed using the β -hydroxybutyrate LiquiColor[®] procedure no. 2440-058 (Stanbio Laboratories, Boerne, TX, USA). Each analysis was done in duplicate and the average of the two samples was used.

Dry matter intake for each cow was determined daily from 2 weeks prepartum until 5 weeks postpartum. Weekly mean DMI was calculated and used in the final analysis.

2.6. Statistical analysis

Data were analyzed by repeated measures using PROC MIXED procedure of the Statistical Analysis System (Statistical Analysis Systems Institute, Inc., Software version 9.0, 1999–2001). For the final analysis, cows were assigned to one of three uterine health categories (metritis, subclinical endometritis, or normal uterine health) based on the results of uterine health examinations. The model used included the fixed effects of time (week relative to calving), disease (presence or absence of metritis or SC endometritis), time \times disease interaction, the random effect of cows nested within disease, and the residual error. For each variable analyzed (PMN myeloperoxidase activity, cytochrome *c* reduction, DMI, plasma NEFA and BHBA concentration), cow nested within treatment was subjected to three covariance structures: compound symmetry, autoregressive order 1, and unstructured covariance. The covariance that resulted in the Akaike's information criterion closest to zero was used (Littell et al., 1998). In all cases it was the autoregressive order 1 covariance structure. Means and S.E.M. are reported for all data. When significant effects ($P < 0.05$) due to disease, time, or disease \times time interactions were detected, means separation was conducted by the Tukey–Kramer option in SAS 9.1.

Relationships between PMN myeloperoxidase activity and PMN cytochrome *c* reduction across all time points and between PMN myeloperoxidase activity and plasma NEFA during the week of calving were analyzed using linear regression in SAS 9.1.

3. Results

3.1. PMN function and uterine health disorders

Of 83 cows, 18 (21.7%) were diagnosed with puerperal metritis, 43 (51.8%) were diagnosed with subclinical endometritis, 13 (15.7%) were diagnosed with endometritis, and 22 (26.5%) had normal uterine health. All cows with endometritis were included in either the SC endometritis or puerperal metritis categories and therefore were not included in a separate category. Cows with puerperal metritis (occurring between 0 and 14 days after parturition) and SC endometritis (diagnosed at 28 ± 3 days postpartum)

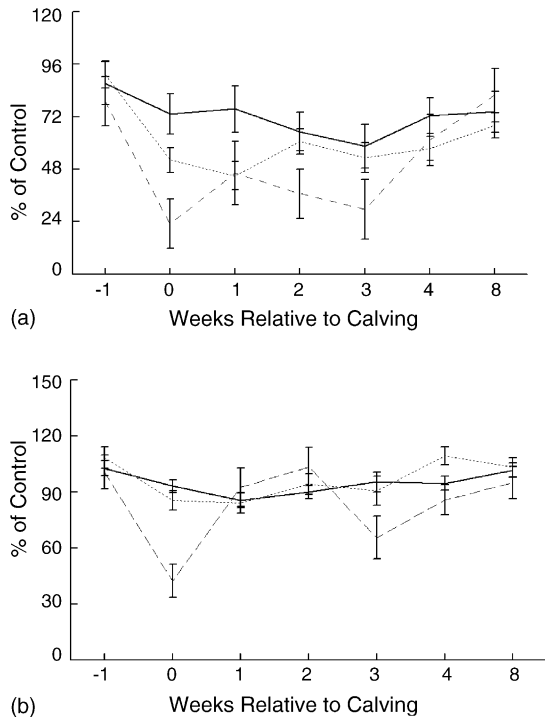


Fig. 1. Myeloperoxidase activity of blood neutrophils (A) and cytochrome *c* reduction of blood neutrophils (B) from dairy cows classified as having puerperal metritis (---, $n = 18$), subclinical endometritis (···, $n = 43$) or normal uterine health (—, $n = 22$). Cytochrome *c* reduction and myeloperoxidase activity shown is the percentage of response of neutrophils obtained from subject animals, compared to the response of neutrophils obtained from the internal laboratory standard steers samples obtained at the same time. Values shown are mean \pm S.E.M.

had significantly lower ($P < 0.05$, disease effect, disease \times time) PMN myeloperoxidase than cows with normal uterine health activity beginning prior to parturition and extending through the early postpartum period. Cows with puerperal metritis had significantly lower ($P < 0.05$, disease effect) PMN cytochrome *c* reduction around the time of calving compared to cows with SC endometritis and cows with normal uterine health (Fig. 1). Neutrophil myeloperoxidase activity and cytochrome *c* reduction activity assayed on all blood PMN within all cows across all time points were positively correlated ($R = 0.4$, $P < 0.01$).

3.2. PMN function, NEFA and DMI

Of the 83 cows, 20 (24.1%) had elevated NEFA levels prior to parturition. Both neutrophil myeloper-

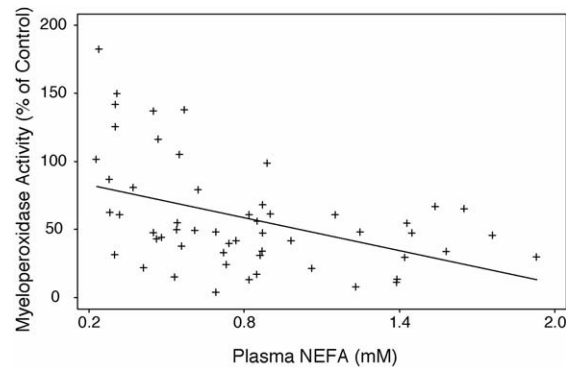


Fig. 2. Relationship between PMN myeloperoxidase activity and plasma nonesterified fatty acid (NEFA) concentration ($R = 0.44$, $P < 0.001$).

oxidase activity and NEFA concentrations were determined for 55 of the 83 cows at the time point corresponding to the first week after calving. In this set of cows, neutrophil myeloperoxidase activity and NEFA concentrations during the first week after calving were negatively correlated within the same cow ($R = 0.44$, $P < 0.01$, Fig. 2). Cows with elevated levels of NEFA prior to parturition (week -2 and -1) had significantly lower ($P < 0.02$, disease \times time interaction) PMN myeloperoxidase activity compared to cows without elevated NEFA during the week prior to parturition (Fig. 3).

Cows in the lowest quartile for prepartum DMI (average daily DMI from 2 weeks before until the week of parturition) had significantly ($P < 0.01$) lower PMN myeloperoxidase activity compared to cows in the highest quartile for prepartum DMI (Fig. 4). Neutrophil myeloperoxidase activity in cows with low prepartum DMI was suppressed prior to parturition, declined through week 1, and remained suppressed until week 3. In contrast, PMN myeloperoxidase activity in cows with high prepartum DMI was high prior to parturition, relative to cows with poor prepartum DMI, and declined only slightly around the time of parturition.

3.3. DMI, NEFA, BHBA and uterine health disorders

Cows with puerperal metritis or SC endometritis had significantly ($P < 0.001$, disease effect) lower DMI beginning 1 week prior to parturition, compared

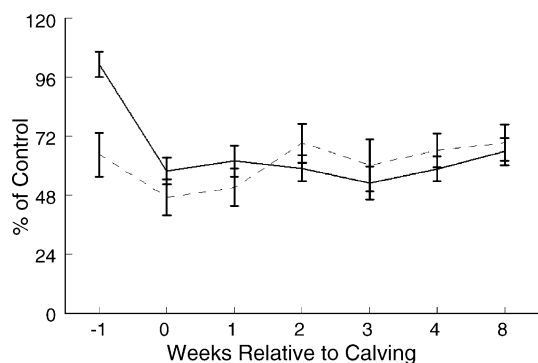


Fig. 3. Myeloperoxidase activity of neutrophils from dairy cows with elevated non-esterified fatty acids (NEFA) (>0.4 mequiv./l) prior to parturition (dashed line, $n = 20$) compared to cows without elevated NEFA prior to parturition (solid line, $n = 63$). Myeloperoxidase activity shown is the percentage of response of neutrophils obtained from subject animals, compared to the response of neutrophils obtained from the internal laboratory standard steers samples obtained at the same time. Values shown are mean \pm S.E.M.

to cows with normal uterine health (Fig. 5A). Cows with puerperal metritis or SC endometritis had significantly ($P = 0.01$, disease effect) higher NEFA levels (Fig. 5B) beginning 2 weeks prior to parturition and significantly higher ($P < 0.05$, disease effect) BHBA levels weeks 1–4 after parturition (Fig. 5C), compared to cows with normal uterine health.

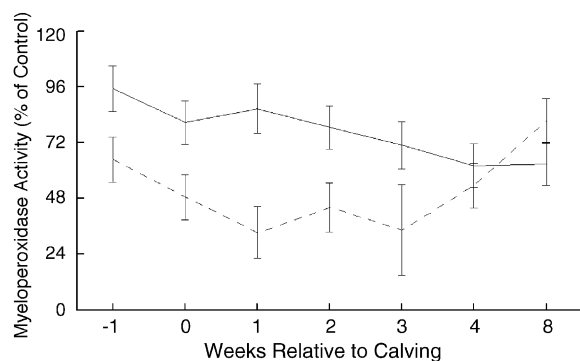
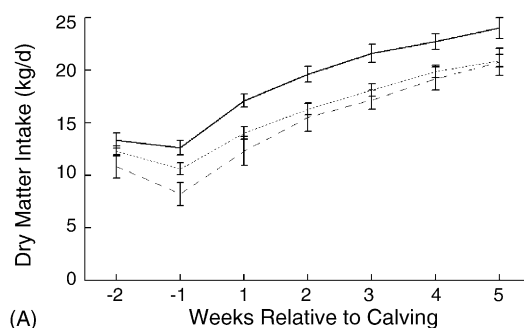
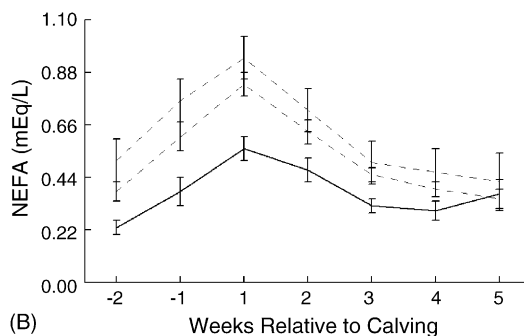


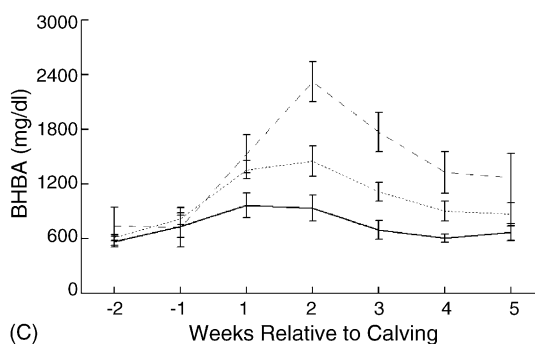
Fig. 4. Myeloperoxidase activity of blood neutrophils from cows within the highest quartile (solid line) of prepartal dry matter intake (DMI) compared to cows within the lowest quartile (dashed line) of dry matter intake during the 3 week period prior to parturition. Myeloperoxidase activity shown is the percentage of response of neutrophils obtained from subject animals, compared to the response of neutrophils obtained from the internal laboratory standard steers samples obtained at the same time. Indicates values for cows in lowest quartile DMI are significantly ($P < 0.05$) lower than for cows in highest quartile DMI. Values shown are mean \pm S.E.M.



(A)



(B)



(C)

Fig. 5. Dry matter intake (A), plasma non-esterified fatty acid concentration (B, NEFA), and plasma β -hydroxybutyric acid concentration (C, BHBA), from dairy cows classified as having puerperal metritis (---, $n = 18$), subclinical endometritis (···, $n = 43$) or normal uterine health (—, $n = 22$). Values shown are mean \pm S.E.M.

4. Discussion

The postpartum uterus in dairy cows is susceptible to multiple bacterial pathogens and susceptibility to infection appears to be associated with periparturient immunosuppression and energy status. Neutrophils play an important role as they provide the first-line of cellular defense against bacterial colonization within the uterus. Previous studies indicate that bovine PMN

functions begin to decline 3–5 weeks prior to parturition, reach a nadir between calving and one week postpartum, and slowly return to prepartum levels 2–4 weeks postpartum (Kehrli et al., 1989; Kimura et al., 1999; Detilleux et al., 1995; Politis et al., 1996). Although impairment of PMN function in periparturient dairy cows has been established, factors associated with PMN impairment around the time of calving are largely unknown.

PMN cytochrome *c* reduction and myeloperoxidase activity following ingestion of opsonized particles were used as measures of PMN ability to kill bacteria. These PMN function assays were chosen because they are well established and considered reliable measures of PMN killing ability. In the present study, the two measures of PMN killing ability, blood PMN myeloperoxidase activity and cytochrome *c* reduction, were significantly correlated within cow when determined simultaneously on the same blood sample. Blood PMN myeloperoxidase activity and cytochrome *c* reduction in cows with normal uterine health generally decreased only slightly around the time of parturition, then remained relatively stable during the remainder of the sampling period. In contrast, in cows with puerperal metritis or SC endometritis PMN myeloperoxidase activity decreased sharply prior to parturition (Fig. 1A). Cytochrome *c* reduction also declined sharply prior to parturition in cows with puerperal metritis, but not in cows with SC endometritis and normal uterine health (Fig. 1B). These results are in general agreement with Cai et al. (1994) who showed that cows with metritis had decreased cytochrome *c* reduction activity prior to parturition, compared to clinically normal cows. However, Cai et al. (1994) reported that PMN myeloperoxidase activity declined only after parturition; whereas the decline occurred prior to parturition in our study. Cai et al. (1994) also reported a decline in circulating PMN after parturition, but not before, in cows with metritis compared to normal cows. Numbers of circulating PMN were not recorded in the present study. Zerbe et al. (2002) reported that PMN from uterine lochia (but not PMN from blood) of cows with endometritis caused by *E. coli* and *Arcanobacterium pyogenes* had altered phenotype and decreased antibody-independent cellular cytotoxicity, compared to healthy cows. This is the first report of declining PMN myeloperoxidase activity

occurring in cows prior to parturition in cows that would develop metritis and the first report linking PMN impairment in periparturient cows prior to or at calving with development of SC endometritis 3–4 weeks later.

The mechanisms responsible for PMN function impairment in periparturient dairy cows are poorly understood. The metabolic challenges associated with late gestation and the onset of lactation could be responsible in part for PMN function impairment during this time (Kimura et al., 1999). The present data suggest an association between energy status prior to calving and PMN function impairment in periparturient dairy cows. Elevation of NEFA (Fig. 3) and suppression of DMI (Fig. 4) prior to parturition were associated with suppressed PMN myeloperoxidase activity, but not cytochrome *c* reduction activity, during the periparturient period. Blood PMN myeloperoxidase activity and plasma NEFA concentration in the days around calving were negatively correlated (Fig. 2), suggesting that cows experiencing negative energy balance prior to or around calving are predisposed to periparturient immune suppression. The myeloperoxidase activity of PMN was generally more dramatically affected than was cytochrome *c* reduction activity by periparturient insults to neutrophil function. Though the two assays both measure neutrophil functions, there are some critical differences in the physiologic functions assessed. The ability of PMN to reduce cytochrome *c* is dependent on adequate production of superoxide anion. Failure to recognize the opsonized zymosan, or failure to stimulate the oxidase enzymes located on the cell membrane and inner surface of the phagosomal membrane could result in poor cytochrome *c* reduction scores. A lack of glycogen stored within the neutrophil may also limit the production of NADPH by the hexose monophosphate shunt. NADPH must be available if the neutrophil is to reduce molecular oxygen to superoxide anion. Poor PMN iodination activity can result from failure to recognize and ingest the opsonized zymosan, a lack of oxidative metabolism within the cell (poor cytochrome *c* reduction activity), failure of the neutrophil to degranulate, a reduced amount of myeloperoxidase within primary granules, destruction of hydrogen peroxide by tissue catalyzes, or interference with myeloperoxidase activity. Because a successful

iodination reaction is dependent on a more complex chain of events than the cytochrome *c* reduction reaction, it tends to be a less specific but more sensitive indicator of disruption of PMN function.

The results of the present study differ from the study of [Stable et al. \(2003\)](#) who reported no difference in PMN myeloperoxidase activity between prepartum cows with decreased DMI and cows that were stuffed through ruminal canulas to maintain DMI, despite the observation that cows allowed to express the typical decline in prepartum DMI had elevated NEFA levels, which was prevented by stuffing the cows with feed. However, [Hoeben et al. \(1997\)](#) reported that subketotic concentrations of BHBA significantly reduced PMN respiratory burst activity in vitro, as measured by a chemiluminescence assay, but elevated BHBA had no effect on either myeloperoxidase activity or cytochrome *c* reduction. To our knowledge, this is the first report linking impaired PMN function with suppression of DMI and elevation in NEFA prior to parturition.

Previous studies have reported a relationship between negative energy balance accompanied by elevated ketone levels during early lactation, and periparturient diseases, including metritis ([Erb and Grohn, 1988](#); [Grohn et al., 1989](#); [Correa et al., 1993](#)). [Hill et al. \(1985\)](#) showed an association between accumulation of lipids in liver and increased length of bacterial shedding in cows with mastitis. [Kaneene et al. \(1997\)](#) reported that prepartum fat mobilization and serum lipoprotein metabolism were related to increased risk of metritis and RP. In contrast, [Jorritsma et al. \(2000\)](#) reported no difference in the incidence of endometritis in cows with high (>50 mg/g) and normal liver triacylglycerol contents. However, in a study reported by [Jorritsma et al. \(2000\)](#) endometritis was diagnosed by rectal palpation and not by endometrial cytological examination as reported in this study. [Reist et al. \(2003\)](#) reported that elevated milk acetone concentrations, but not serum BHBA, were associated with an increased risk for endometritis diagnosed using vaginoscopy. Although there appears to be an association between negative energy balance in early lactation and infectious diseases during early lactation, the underlying mechanisms for this association remain unclear. Our study supports and advances these observations by demonstrating that

metabolic disturbances occurring before calving predispose cows to later uterine health disorders. This is the first report describing an association between decreased prepartal DMI and elevated prepartal plasma NEFA concentration with subsequent development of SC endometritis after calving.

In summary these data suggest that some uterine health disorders are associated with impairment of PMN function and negative energy status that begins prior to calving and extends into early lactation. Furthermore, our results provide evidence that impaired PMN function around the time of parturition is associated with nutrient deficiencies that occur prior to parturition.

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